

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re Application of: |) Atty. Docket No.: STURK0003 |
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| Ralf RESKI et al. |) Confirmation No. 9421 |
| |) |
| Serial No. 10/089,450 |) Group Art Unit: 1638 |
| |) |
| Filed: March 29, 2002 |) Examiner: KUBELIK, Anne R. |
| |) |
| For: METHOD FOR THE PRODUCTION |) |
| OF PROTEINACEOUS |) |
| SUBSTANCES |) |

DECLARATION UNDER 37 C.F.R. § 1.132

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1. I, Ralf Reski, state that I am an expert in the field of plant cell transformation and cultivation research and development. A copy of my Curriculum Vitae is attached herewith as evidence of my relevant expertise. I am also recognized by other

researchers in the field of *Physcomitrella patens* molecular biology for my work in accumulating a database of >100,000 ESTs for *Physcomitrella patens* (See Tomoaki Nishiyama et al., *Comparative genomics of Physcomitrella patens gametophytic transcriptome and Arabidopsis thaliana: Implication for land plant evolution*, 100 PNAS 8007-8012 (2003), at <http://www.pnas.org/cgi/content/full/100/13/8007>, (a downloaded copy is attached hereto). Furthermore, I am member of the international Moss Genome Consortium.
<http://www.jgi.doe.gov/sequencing/why/CSP2005/physcomitrella.html>.

2. I am familiar with the above-captioned patent application and claims. In this declaration, I review relevant literature regarding bryophytes, in particular mosses of the species *Physcomitrella*, *Ceratodon* and *Funaria*, and liverworts of the species *Marchantia*, and provide my testimony regarding the predictability of cellular transformation in view of a particular enabled cellular transformation in the moss species *Physcomitrella patens*. In this declaration, I also review experimental evidence demonstrating the predictability, and ease, of transforming and cultivating transgenic *Funaria hygrometrica* (a moss) and transgenic *Marchantia polymorpha* (a liverwort). In this declaration, I also provide my testimony regarding the so-called

Wands factors, which, I am informed are factors considered in the determination of whether a U.S. patent application is enabled.

3. The method for the production of heterologous proteinaceous substances in plant material, in accordance with the present invention, as defined by claims 1-6, 17 and 19-21 of the above captioned patent application, includes the steps of (a) culturing plant material in a culture medium, wherein the plant material is protonema tissue, transformed with a construct encoding a signal peptide operably linked to a protein, that produces heterologous proteinaceous substances, and (b) obtaining secreted heterologous proteinaceous substances from the culture medium without disrupting producing tissues or cells. In interpreting the term “protonema,” a person of ordinary skill in the art would realize that “protonema” pertains to “the primary [usually] filamentous thalloid stage of the gametophyte in mosses and some liverworts” as defined in Webster’s new collegiate dictionary, 1977, at 927 (of record). Therefore, the “plant material” employed in the method of the present invention is protonema tissue, which pertains to the primary usually filamentous thalloid stage of the gametophyte in mosses and some liverworts.

Literature Considered

4. In preparation for rendering an opinion regarding the scope of enablement of the above-captioned patent application, I reviewed the specification of U.S. Patent Application No. 10/089,450 and the following relevant literature:

- a. *Bryophyte: species list*, COLLECTIONS, Department of Cryptogamic Botany, Swedish Museum of Natural History, at <http://www2.nrm.se/kbo/saml/mossor/p.html.en> (hereafter, the “Bryophyte Species List”) (of record);
- b. B. Goffinet, *Physcomitrella*, BRYOPHYTE FLORA OF NORTH AMERICA, Provisional Publication, at <http://www.mobot.or/plantscience/BFNA/V1/FunaPhyscomitrella.htm> (Sept. 13, 2005) (hereafter, the “Physcomitrella reference”) (of record);
- c. Nichole Houba-Hérin et al., *Cytokinin oxidase for Zea mays: purification, cDNA cloning and expression in moss protoplasts*, 17 THE PLANT JOURNAL 615 (hereafter, the “Houba-Hérin article”) (of record);
- d. K. Reutter and R. Reski, *Production of a heterologous protein in bioreactor cultures of fully differentiated moss plants*, 2 PLANT TISSUE CULTURE AND BIOTECHNOLOGY 142 (1996) (hereafter, the “Reutter article”) (of record);
- e. M. Nasu et al., *Efficient transformation of Marchantia polymorpha that is haploid and has very small genome DNA*, 84 J. FERM. BIOENGINE. 519 (1997) (hereafter, the “Nasu article”) (of record);

- f. M. Zeidler et al., *Transgene expression in the moss Ceratodon purpureus*, 154 J. PLANT PHYSIOL. 641 (1999) (hereafter, the “Zeidler article”) (of record)
- g. Schaefer et al., *Stable transformation of the moss, Physcomitrella patens*, 226 MOL. GEN. GENET. 418 (1991) (hereafter, the “Schaefer article”) (of record);
- h. F. Thümmmler et al., *Expression of oat phyA cDNA in the moss Ceratodon purpureus*, 56 PHOTOCHEM. PHOTOBIOLOG. 771 (1992) (hereafter, the “Thümmmler article”) (of record);
- i. Sawahel et al., *Transfer of foreign DNA into Physcomitrella patens protonemal tissue using the gene gun*, 10 PLANT MOL. BIO. REP. 314 (1992) (hereafter, the “Sawahel article”) (of record);
- j. H. Mühlbach, *Use of plant cultures in biotechnology*, 4 BIOTECHNOLOGY ANNUAL REVIEW 113 (1998) (hereafter, the “Mühlbach article”) (of record); and
- k. R. Reski, *Development, Genetics and Molecular Biology of Mosses*, 111 BOT. ACTA 1 (1998) (hereafter, the “Reski article”) (of record).

Review of the Literature

5. In view of my review of the above relevant literature, and based upon my knowledge, experience and belief, I testify to the following. I believe there are currently about 20,000 bryophytes that have been taxonomically described, and of these 20,000 bryophytes only 5 subspecies are taxonomically described as

Physcomitrella (See the Bryophyte Species List) and have been transferred to axenic cultivation. *Physcomitrella* species/subspecies share certain phenotypic characteristics, such as an immersed capsule with irregular dehiscence, and thin-walled, translucent exothecal cells (See the *Physcomitrella* Reference). Although mainly *Physcomitrella patens* has been used for broad investigations pertaining to genetics and physiology, this is not to say that a person of ordinary skill in the art would not expect other *Physcomitrella* species/subspecies to behave in the same way in the same transgenic experiments as *Physcomitrella patens*. Likewise, there is no reason why a person of ordinary skill in the art would not expect other *Physcomitrella* species/subspecies to behave in the same way under the same cell culture conditions as *Physcomitrella patens*.

6. On the other hand, in my opinion, a person of ordinary skill in the art would reasonably predict members of the *Physcomitrella* species to behave in a manner similar to *Physcomitrella patens*. I have reviewed the additional evidence submitted by Dr. Gilbert Gorr in the Declaration under 37 C.F.R. § 1.132 (hereafter, the “Gorr Declaration”), filed December 7, 2005. In my opinion, the Gorr Declaration, ¶¶ 14-23, demonstrates that more remote bryophyte relatives, such as *Ceratodon* species and *Marchantia* species can also be transformed and cultivated under the same conditions

as *Physcomitrella patens*. In view of such evidence, which I discuss below, in my opinion a person of ordinary skill in the art would expect the 4 other members of the *Physcomitrella* species to undergo transformation and cultivation under the same conditions as *Physcomitrella patens*. In other words, in my opinion, a person of ordinary skill in the art would expect mosses of the species *Physcomitrella* to be predictably employable in the method of the present invention for the reasons that follow.

The Bryophyte Culture Technology

7. The relevant literature teaches that while the scientific community working with bryophytes placed special emphasis on developing protocols for the genetic transformation and cultivation under photoautotrophic conditions of *Physcomitrella patens*, protocols were developed for other bryophytes as well. In fact, a broad spectrum of bryophytes can be cultivated under similar photoautotrophic conditions. For example, as described in the instant U.S. Patent Application No. 10/089,450, a protocol has been developed for the cultivation of *Physcomitrella patens* (See Specification, page 12, lines 13-29, and page 17, lines 1-34). However, similar photoautotrophic cultivation conditions have been described for culturing *Ceratodon purpureus* (a moss) and for culturing *Marchantia polymorpha* (a liverwort), (See the

Zeidler article and the Nasu article, respectively). Relevant scientific literature demonstrates that bioreactor cultivation techniques have been developed for a wide variety of mosses and liverworts.

8. Based on my knowledge and experience in the field of bryophyte cell transformation and cultivation, and based on my review of the relevant scientific literature, it is my opinion that a person of ordinary skill in the art would be able to adapt the known culture protocols for *Physcomitrella patens*, *Ceratodon purpureus*, *Marchantia polymorpha*, etc. to cultivate other bryophytes under similar photoautotrophic conditions. It is further my opinion that any such modification of known culture protocols in order to optimize growth of other bryophyte species (i.e., *Physcomitrellas*, *Ceratodons*, *Marchantia*, *Funaria*, *Sphagnum*, *Sphareroocarpos*, and others) in a bioreactor would take no more than routine experimentation because the technology is well established and it is not difficult to grow mosses and liverworts in culture (See e.g. J. Hata et al. (1999), Photoantotrophic cultures of the host and transformed cells of *Marchantia polymorpha* under controlled incident light intensity, J. Biosci. Bioeng., 88(5): 582-5, abstract enclosed) .

The Isolation of Protoplasts

9. The isolation of protoplasts is a prerequisite for cellular transformation as described on page 18, lines 28-35, of the specification of the above-captioned U.S. Patent application No. 10/089,450. The isolation of, and cellular transformation of, *Physcomitrella patens* protoplasts has been reported and reliably established (See Schaefer article, generally; and Mühlbach article, at 160-161), although the recovery of heterologous proteinaceous substances from protonema tissue without disrupting producing tissues or cells has not been previously reported. Review of the scientific literature demonstrates that isolation protocols for protoplasts of many other species of bryophytes, such as *Ceratodon purpureus* (See Thuemmler article; and Zeidler article), *Funaria hygrometrica* (See Mejia article), and liverworts (See Nasu article), are also well established.

10. Thus, based on my knowledge and experience, and based on my review of relevant scientific literature, it is my opinion that a person of ordinary skill in the art would expect the isolation of additional bryophyte species, besides *Physcomitrella patens*, to be easily achieved by adapting known pre-existing isolation protocols. Furthermore, based on my knowledge and experience, it is my opinion that a person of ordinary skill in the art would have to employ no more than routine

experimentation to adapt the pre-existing protoplast isolation protocols to other bryophyte species.

Genetic Transformation of Bryophyte Species

11. The genetic transformation of a wide variety of bryophyte species is well known and can be done successfully using many different techniques. In particular, the genetic transformation of *Physcomitrella patens* using PEG-mediated DNA-transfer into protoplasts, as described on page 13, line 9, to page 14, line 7, and on page 18, lines 28-35, of the specification of the instant U.S. Patent Application No. 10/089,450, is one example. Other PEG-mediated DNA-transfer protocols for *Physcomitrella patens* have been described in the Schaefer article and the Sawahel article, as well as for other species of bryophytes (i.e., for transformation of *Ceratodon purpureus*, see the Thuemmler article and the Zeidler article).

12. Other transformation protocols exist and for a review of the molecular biology of mosses see the Reski article, at 9-10. For example, the Sawahel article teaches biolistic transformation of *Physcomitrella patens*, and the Nasu article teaches Argobacterial mediated transformation of *Marchantia polymorpha*. As taught by the Zeidler article, different bryophyte species may require slightly different

transformation conditions, e.g., different promoters may be useful and applicable. Thus, in view of my own knowledge and experience, and in view of my review of relevant scientific literature, it is my opinion that the state of the art, at the time the invention was made, would have enabled a person of ordinary skill in the art to apply known genetic transformation methods to different bryophyte species, besides *Physcomitrella patens*, and that the application of these known genetic transformations to other bryophyte species would involve no more than routine experimentation.

Summary of Relevant Scientific Literature

13. In summary, the relevant scientific literature demonstrates that genetic cellular transformation protocols for various bryophyte species, in particular, *Physcomitrella patens*, *Ceratodon purpureus*, and *Marchantia polymorpha*, are well known. While these are only three specific species of bryophytes, there is no reason to believe that other subspecies and/or species of bryophytes would not be amenable to known cellular transformation techniques, especially since bryophytes are relatively primitive, simple and well-characterized plants. However, since the present invention is only indirectly related to the genetic transformation of bryophytes, it is my opinion that the state of the art of bryophyte genetic transformation is sufficiently developed,

well-characterized and mature to enable a person of ordinary skill in the art to make and use the presently claimed invention without undue experimentation, which pertains to the culturing of transformant bryophyte protonema tissue, and to obtaining heterologous proteinaceous substances from the culture medium employed in the culturing step.

Experimental Evidence Provided by the Gorr Declaration

14. The additional experiments reported by the Gorr Declaration support my above conclusions (Gorr Declaration, 14-23). These additional experiments show (1) that the genetic transformation protocol described in U.S. Patent Application No. 10/089,450 are applicable to other bryophyte species (i.e., *Funaria hygrometrica* (moss) and *Marchantia polymorpha* (liverwort)), without undue experimentation, (2) that the cell culture protocol described in U.S. Patent Application No. 10/089,450 is applicable to other bryophyte species (i.e. mosses such as *Funaria hygrometrica*, *Ceratodon purpureus*, *Pylaisia selwynii* and *Pylaisia polyantha*, and liverworts such as *Marchantia polymorpha* and *Jungermania leiantha*), without undue experimentation, and (3) that heterologous proteinaceous substances produced from other transformant protonema tissue (i.e., *Funaria hygrometrica* and *Marchantia polymorpha*) may be obtained from the culture medium.

Transformation of Additional Bryophytes

15. Using the PEG-mediated DNA-transfer protocol described on page 14, line 1, to page 16, line 7, and on page 16, lines 28-35, of U.S. Patent Application No. 10/089,450, which employs the transformation protocol of the Reutter article, Dr. Gorr isolated protoplasts of the moss *Funaria hygrometrica* that were successfully and stably transformed (Gorr Declaration, ¶¶ 14-16). In Dr. Gorr's study of *Funaria hygrometrica*, the plasmid pRT99 containing the coding sequence for NPTII under regulation of promoter 35S was used, which is the same plasmid used in the example provided in U.S. Patent Application No. 10/089,450 for *Physcomitrella patens* transformation, and the marker used for the selection procedure was neomycin phototransferase II (NPTII), which mediates resistance to the antibiotic G418, thereby permitting selection of successfully transformed clones such as shown by Figures 1(a) and 2 of the Gorr Declaration (Gorr Declaration, Declaration, ¶¶ 14-16).

16. Using the PEG-mediated DNA-transfer protocol described on page 14, line 1, to page 16, line 7, and on page 16, lines 28-35, of U.S. Patent Application No. 10/089,450, which employs the transformation protocol of the Reutter article, Dr. Gorr isolated protoplasts of the liverwort *Marchantia polymorpha* that were

successfully and stably transformed (Gorr Declaration, ¶¶ 14 and 17). In Dr. Gorr's study of *Marchantia polymorpha*, the plasmid pRT99 containing the coding sequence for NPTII under regulation of promoter 35S was also used, thereby permitting selection of successfully transformed *Marchantia polymorpha* clones such as shown by Figures 1(b) and 2 of the Gorr Declaration (Gorr Declaration, Declaration, ¶¶ 14 and 17).

17. In my opinion, the bryophyte clone transformation results provided by the Gorr Declaration, which were obtained without undue experimentation, support the conclusion that the transformation of additional bryophyte species and subspecies can be achieved by using known genetic transformation protocols under conditions employing no more than routine experimentation encountered in the art.

Culturing of Additional Bryophytes

18. Using the cultivation conditions described on page 17, line 1, to page 18, line 26, of U.S. Patent Application No. 10/089,450, Dr. Gorr demonstrated that additional moss and liverwort species were successfully cultivated under the same liquid culture conditions, the same Knop medium, and the same light intensities, as described in the above-captioned application for cultivating *Physcomitrella patens* (Gorr Declaration,

¶ 19). The additional bryophyte species that were successfully cultured, employing no more than routine experimentation, include the mosses *Funaria hygrometrica*, *Ceratodon purpureus*, *Pylaisia selwynii* and *Pylaisia polyantha*, and liverworts *Marchantia polymorpha* and *Jungermania leiantha*. In my opinion, the bryophyte culture results provided by the Gorr Declaration, which were obtained without undue experimentation, support the conclusion that applying the culture protocol described in U.S. Patent Application No. 10/089,450 to additional bryophyte species and subspecies can be achieved under conditions employing no more than routine experimentation encountered in the art.

Obtaining Recombinant Proteins From Additional Transformant Bryophytes

19. Using the PEG-mediated DNA-transfer protocol described on page 14, line 1, to page 16, line 7, and on page 16, lines 28-35, of U.S. Patent Application No. 10/089,450, which employs the transformation protocol of the Reutter article, Dr. Gorr isolated protoplasts of the moss *Funaria hygrometrica*, and the liverwort *Marchantia polymorpha*, that were successfully and stably transformed so as to recombinantly express and secrete human vascular endothelial growth factor (rhVEGF) (Gorr Declaration, ¶¶ 21-23). In Dr. Gorr's study, the plasmid pRT99 containing the coding sequence for rhVEGF under regulation of promoter 35S was

used, which is the same plasmid used in the example provided in U.S. Patent Application No. 10/089,450 for *Physcomitrella patens* transformation (Gorr Declaration, ¶ 21). In my opinion, Dr. Gorr's transformation of *Funaria hygrometrica* and *Marchantia polymorpha* bryophytes required no more than routine experimentation, as is commonly encountered in the art.

20. In my opinion, Dr. Gorr also demonstrated that stable transgenic strains of *Funaria hygrometrica* and *Marchantia polymorpha* bryophytes successfully express and secrete rhVEGF into the medium of liquid cultures, as shown in Figure 2 of the Gorr Declaration. Figure 2 of the Gorr Declaration illustrates ELISA quantification of secreted rhVEGF in supernatants of transgenic strains of *Funaria hygrometrica* protonema and *Marchantia polymorpha* protonema, where concentrations of secreted rhVEGF measured in crude supernatants were in ranges of 2 to 10 ng/ml for both the transgenic *Funaria hygrometrica* strain and the transgenic *Marchantia polymorpha* strain (Gorr Declaration, ¶22). In my opinion, Figure 2 of the Gorr Declaration clearly shows that transgenic strains of *Funaria hygrometrica* and *Marchantia polymorpha* are suitable for use in the present invention. It is also my opinion, based on the results reported in the Gorr Declaration, that suitable production of rhVEGF by transgenic strains of *Funaria hygrometrica* protonema and *Marchantia polymorpha*

protonema is achievable, and that the method of the presently claimed invention was sufficiently described at the time of filing to enable a person of ordinary skill in the art to make and use the invention without undue experimentation.

Analysis of the Wands Factors

21. In view of my analysis of the relevant literature in the art, my analysis of the experimental data provided by the Gorr Declaration, and based on my own knowledge and experience in the art, I offer the following testimony and opinion regarding the so-called Wands factors.

Direction and Guidance from Specification

22. In my opinion, the present specification gives ample guidance regarding how to practice and use the claimed invention as recited in claims 1-6, 17 and 19-21. Specifically, the specification provides ample guidance regarding how to use intact plants to obtain secreted heterologous proteinaceous substances directly from the culture medium without disrupting producing tissues or cells (See specification, page 9, line 5, to page 27, line 26). In other words, the present specification provides about 18 pages of detailed instruction. Also, in my opinion, the starting materials for practicing the methods are known in the prior art and are commercially available (see

page 7, lines 1-8, and page 9, lines 6-24), with materials described for a detailed example on page 11, line 29, to page 26, line 18, of the present specification. The specification further describes multiple species of mosses and liverworts that are well-known, characterized, and previously studied and that are suitable for practicing the present invention, including *Physcomitrella*, *Funaria*, and *Ceratodon* species (See specification, page 9, lines 6-24, and R. Reski, *Development, genetics and molecular biology of mosses*. 111 Bot. Acta 1, 1-15 (1998) (of record), hereafter the “Reski article”) as well as *Sphagnum* species (See specification, page 9, lines 6-18, and H. Rudolph and S. Rasmussen, *Studies on secondary metabolism of Sphagnum cultivated bioreactors*. 3 Crypt. Bot. 67, 67-73 (1992) (of record), hereafter the “Rasmussen article”).

23. In my opinion, the specification gives detailed direction and guidance regarding the genetic transformation of a single protonema forming species *Physcomitrella patens* so that the protonema tissue produces vascular endothelial growth factor VEGF (See specification as originally filed, page 11, line 29, to page 18, line 35). The specification explains that genetic transformation systems for *Physcomitrella patens* have been previously developed and described for enzyme production (See specification, page 9, line 26, to page 10, line 2, and K. Reutter and

R. Reski, *Production of heterologous protein in bioreactor cultures of fully differentiated moss plants*. 2 Plant Tissue Culture and Biotechnology 142, 142-147 (1996) (of record)).

24. In view of the facts demonstrating the application of genetic transformation techniques to multiple species of plant protonema tissue, it is my opinion that a person of ordinary skill in the art would only have to apply routine experimentation to adapt the method described by the specific example provided by the instant specification, wherein the plant protonema tissue is *Physcomitrella patens*, to other bryophyte species developing protonema tissue whether belonging to mosses or liverworts. My opinion is based on the direction and guidance provided by the disclosure of the above-captioned application, and is based on my knowledge and experience in the art, and is bolstered by the fact that when such additional experiments are carried out by those of ordinary skill in the art, successful transformation of other species of mosses and liverworts can be achieved without undue experimentation (See Gorr Declaration, ¶¶ 18 and 30).

25. In my opinion, for all of the above reasons, the first Wands factor, direction and guidance from the specification, weighs in favor of enablement.

There is a Working Example Present

26. Based on my personal knowledge and belief, the present specification provides a specific enabling example in the disclosure to teach how to perform and use the “method for the production of heterologous proteinaceous substances in plant material...wherein the plant material is protonema tissue” as described on page 11, line 29, to page 18, line 35 of the specification as originally filed. In my opinion, protonema tissue is a well-known plant tissue type, which manifests numerous common biological characteristics and functions whether originating from moss or liverwort species. The Gorr Declaration, ¶¶ 5-22, provides additional support for my opinion.

27. In my opinion, for all of the above reasons, the second Wands factor, the presence of a working example, weighs in favor of enablement.

The Nature of the Invention

28. The present invention is directed to a method of producing heterologous proteinaceous substances using genetically transformed protonema tissue. Thus, the method in accordance with this embodiment of the present invention employs simple

plant organisms that have a well-characterized plant physiology and predictable developmental cycle (See R. Reski, *Development, genetics and molecular biology of mosses*. 111 Bot. Acta 1, 3, 6 and 11 (1998); and the Nasu article, at 519, first column, lines 1-24). In my opinion, based on my knowledge and experience in the art, the biological plant organisms producing protonema tissue, whether of the moss phylum (e.g., *Physcomitrella*, *Funaria*, *Sphagnum* and *Ceratodon*) or the liverwort phylum (e.g., *Marchantia* and *Sphaerocarpos*), are relatively simple, predicatable organisms.

29. In my opinion, for all of the above reasons, the third Wands factor, the nature of the invention, weighs in favor of enablement.

The State of the Art

30. In my opinion, the State of the Art is mature as evident from such prior art references as the Houba-Hérin article, the Reutter article, the Zeidler article, the Nasu article, the Rasmussen article, and the review article by H. Mühlbach, *Use of plant cell cultures in biotechnology*. 4 Biotechnology Annual Review 113, 158-161 (1998) (specifically, page 158, line 32, to page 161, line 6 (of record)) (hereafter, the Mühlbach article). In fact, the Mühlbach article states that

[t]hese studies document the advanced stage that is currently achieved

in the genetic transformation of *P. patens*, which can be certainly extended to other genes and also to other bryophytes with potential use in biotechnology.

Evidence along this line comes from studies on the expression of the human vascular endothelial growth factor (VEGF) protein in bioreactor cultures of *P. patens*....In general, these promising approaches clearly demonstrate the feasibility of bioreactor cultures of transgenic mosses for the production of heterologous compounds. H. Mühlbach, at page 160, line 38, to page 161, line 6, (emphasis added).

In addition, the primary references recited against the claims of the present application, such as Houba-Hérin article, the Reutter article, the Zeidler article and the Nasu article, were published about 5 or more years ago, which further suggests the mature nature of the relevant art. Based on my review of the relevant literature, and based on my own knowledge and experience in the art, I agree with Dr. Gorr (Gorr Declaration, ¶ 30) in concluding that the state of the art regarding the cultivation and transformation of bryophytes, including both mosses and liverworts, was mature and well developed at the time the present application was filed.

31. In my opinion, for all of the above reasons, the fourth Wands factor, the state of the art, weighs in favor of enablement.

The Relative Skill of Those in the Art

32. In my opinion, a person of ordinary skill in the art of transforming plant cells to express selected proteins, such as human VEGF, are highly trained professionals with advanced degrees in cellular and molecular biology, or an equivalent field, who are involved in research and technological advancement of the field. Based on my knowledge and experience in the art, the relative skill level of those in the art is notably high. The Curriculum Vitae of Dr. Gilbert Gorr (of record) is an example, in my opinion, of the level of training and experience that a person of ordinary skill in the art has attained.

33. In my opinion, for all of the above reasons, the fifth Wands factor, the relative skill of those in the art, weighs in favor of enablement.

Predictability of the Art

34. In my opinion, based on my knowledge and experience in the art, the predictability of transformation of moss and liverwort species is high. In addition to the transformability of *Physcomitrella patens* as described in the Schaefer article and the Mühlbach article, the Zeidler article and the Nasu article show that the transformation of other protonema forming species, such as *Ceratodon* and

Marchantia, are also known. Thus, based on available literature, and my experience and knowledge, it is my opinion that the predictability of the art, pertaining to the transformation of plant species that ultimately produce protonema tissue, is highly predictable when compared to fields utilizing viruses, for example. The fact that transformation of moss and liverwort species is highly predictable for a biological art is additionally supported by the transformation results reported by Dr. Gorr (Gorr Declaration, ¶¶ 11-18).

35. In my opinion, for all of the above reasons, the sixth Wands factor, the predictability of the art, weighs in favor of enablement.

Quantity of Experimentation Necessary

36. In my opinion, the present specification generally outlines the method for the production of heterologous proteinaceous substances in protonema tissue in accordance with claims 1-6, 17 and 19-21 of the present invention, as described on page 8, line 1, to page 18, line 35, using known and readily available materials. Based on my knowledge and experience, the crux of the present invention is obtaining secreted heterologous proteinaceous substances from the culture medium without disrupting producing tissues or cells. The method in accordance with these directions

may require some experimentation in order to optimize results using protonema from species other than *Physcomitrella patens*, *Ceratodon purpureus* and *Marchantia polymorpha*; however, it is my opinion that while some experimentation may be necessary, it is no more than is commonly encountered in the art. My opinion is supported by the additional experimental evidence submitted in the Gorr Declaration, which shows successful transformation of *Funaria hygrometrica* was achieved using the protocol described on page 14, line 1, to page 16, line 7, and on page 16, lines 28-35, of U.S. Patent Application No. 10.089,450, and that such successful transformation required no more than routine experimentation as is commonly encountered in the art (Gorr Declaration, ¶¶ 15, 16 and 21-23).

37. It is my opinion that, based on the scientific references of record, the testimony and experimental evidence provided by the Gorr Declaration, and from my own knowledge and experience in the art, a person of ordinary skill in the art would know how to transform *Physcomitrella patens*, *Ceratodon purpureus*, *Funaria hygrometrica* and *Marchantia polymorpha* protoplasts, in view of the disclosure of the above-captioned application and the state of the art at the time the invention was filed, without undue experimentation. Although transformation of plant protoplasts is not the crux of the presently claimed invention, it is my opinion that transformation of

other plant protoplasts of species that produce protonema tissue is a matter of routine experimentation in the art in view of the biological predictability of protonema forming plant tissues in general.

38. In my opinion, for all of the above reasons, the seventh Wands factor, the quantity of experimentation necessary, weighs in favor of enablement.

Breadth of Claims

39. It is my opinion that the breadth of claim 1 includes the method of production of heterologous proteinaceous substances in protonema tissue. The term “protonema” has a specific meaning in the art and limits the scope of the present invention to tissues that are “the primary usually filamentous thalloid stage of the gametophyte in mosses and in some liverworts.” Furthermore, the claimed method includes two steps: (i) culturing transformed protonema tissue in a culture medium to produce heterologous proteinaceous substances and transit peptide for secreting the substances from producing cells, and (ii) obtaining secreted heterologous proteinaceous substances from the culture medium without disrupting producing tissues or cells. The breadth of claim 1, which is the broadest claim, includes the species embodiment

disclosed on page 11, line 29, to page 27, line 26, and is limited to transformed bryophyte strains. The breadth of claim 1, in my opinion, is not overly broad.

40. In my opinion, for all of the above reasons, the eighth Wands factor, the breadth of claims, weighs in favor of enablement.

Specific Testimony Regarding the Houba-Hérin Article

41. I have read and thoroughly reviewed the Houba-Hérin article (of record) and provide the following testimony regarding the scope and content of the teachings of this reference. In my opinion, I agree with Dr. Gorr in that the Houba-Hérin article merely teaches transiently transforming and maintaining *Physcomitrella patens* protoplasts in order to prove the functionality of a certain plant enzyme, as can be taken from the article itself (p. 621, right column, lines 3 to 12) (See Gorr Declaration, ¶ 24). The article does not relate to the production of heterologous substances secreted into the culture medium. Instead, I believe the authors have selected transformed protoplasts because the state of the art related to callus tissues comprising cell walls "...suggested that a compartmentation could exist in the cells that keep the glycosylated form in the cell wall or plasmalemma and the unglycosylated form in an internal compartment" (see p. 621, right column, last para.,

lines 3 to 6). Accordingly, it is my opinion that the authors would not have selected any cell wall comprising plant material, such as protonema tissue. As an additional point, the method employed by the Houba-Hérin article does not integrate heterologous DNA into the genome of the moss protoplasts and I believe a person of ordinary skill in the art would recognize that the Houba-Hérin method was not intended for either long term or commercial enzyme production (See Houba-Hérin article, at 624, second column, lines 35-53) and would not indicate to one of ordinary skill in the art that such was possible.

42. According to the teachings of the Houba-Hérin article, the harvest of medium containing the expressed recombinant protein activity was terminated at about 44 hours following transformation. At this point in time, based on my knowledge and experience in the art, it is my opinion that moss cell wall regeneration generally would not have yet occurred. Therefore, I reach the same conclusion as Dr. Gorr - if any extracellular recombinant protein activity were observed, it would be expressed by moss protoplasts and not by moss protonema tissue (See Gorr Declaration, ¶ 25).

43. In view of my analysis of the teachings of the Houba-Hérin article, it is my opinion that this reference provides no suggestion, hint, or motivation, to a person of

ordinary skill in the art regarding whether successful secretion of the target protein would persist at some later point in time, such as a few days, when cell wall regeneration would normally have occurred as the protoplasts mature into protonema cells and tissue. In other words, it is my opinion that the teachings of the Houba-Hérin article do not provide any suggestion, hint, or motivation that would lead a person of ordinary skill in the art to apply the Houba-Hérin method of transient transformation performed to prove functionality of a given plant enzyme to create transformed bryophyte protonema that would have intact cell walls.

44. Another fact regarding the teachings of the Houba-Hérin article is that the method it employs for detecting enzyme (i.e., cytokinin oxidase (CKO)) is both indirect, because it pertains to detecting levels of CKO enzyme activity rather than the enzyme itself, and is directed to detecting trace levels of enzyme as evident from the photolabeling of cytokinin oxidase (CKO) with [³H]-azidoCPPU (See Houba-Hérin article, at 616, first col., line 26, to second col., line 2). It is my opinion that a person of ordinary skill in the art employs a photolabeling detection method in place of conventional methods of direct measurement, such as the ELISA methods described on page 20, lines 21-27, and page 27, lines 11-26, of the above-captioned application and the ELISA method employed by Gorr (Gorr Declaration, ¶ 22), when the amount

of detectable protein is expected to be in trace amounts, if any is produced at all. The Houba-Hérin article also does not teach the application of a transit peptide, such as is included by the presently claimed invention. As taught by Armin Baur et al., *A fast and flexible PEG-mediated transient expression system in plants for high level expression of secreted recombinant proteins*, 119 J. Biotechnology 332, 337 (2005) (of record), which I co-authored, a target protein that is not associated with a signal peptide is not likely to be secreted in significant amounts from moss protoplasts. This fact would be even more true of moss protonema, which have a cell wall acting as a barrier further limiting secretion. Consequently, it is my opinion that a person of ordinary skill in the art would have no motivation to apply the teachings of the Houba-Hérin article regarding the formation of transiently transformed moss protoplasts for any useful or commercial purpose because the yields of the CKO enzyme are, at most, trace amounts.

45. In my opinion, the presently claimed invention is clearly distinguished from the teachings of the Houba-Hérin article by the fact that the present invention utilizes the protonema tissue and cells of transformed bryophyte protonema. Transformed bryophytes, as described in the specification of U.S. Patent Application No. 10/089,450, are regenerated from single transformed protoplasts that undergo a

selection process (i.e., in view of the G418 resistance conferred by the same transformation). In general, all of the transformed, regenerated bryophyte protonema cells possess cell walls. The cell wall, however, is an important parameter limiting the secretion of heterologous proteins because it provides a major barrier to secretion as is well known in the art. On this point, I agree whole-heartedly with Dr. Gorr (See Gorr Declaration, ¶ 27).

46. I have also reviewed the Reutter article, which discloses the use of stably transformed *Physcomitrella* protonema to intracellularly express a heterologous non-secretory protein (GUS). The plants expressed and accumulated this heterologous protein in the cells. However, as co-author of the Reutter article, I state that no secretion of the heterologous protein into the medium was observed from stably transformed protonema because for secretion of the heterologous protein to occur there has to be at least a signal peptide (i.e., a transit peptide) included with the heterologous protein. The method taught by the Reutter article did not employ a signal peptide. Consequently, this reference neither teaches, nor suggests, there would be secretion of the heterologous protein through the cell wall of protonema cells. The same issue is encountered with the Nasu article, which also does not teach, or suggest, the application of a signal peptide. Consequently, the Nasu article also

neither teaches, nor suggests, there would be secretion of the heterologous protein through the cell wall of protonema cells.

47. I have also reviewed U.S. Patent 6,096,546 to Raskin (hereafter, the “Raskin Patent”), which pertains to methods for recovering polypeptides from higher plants and portions thereof. However, the polypeptides recovered by the Raskin method are obtained by collecting plant exudates, which is fluid that “oozes out” of certain structures of higher order plants such as roots and leaf hydathodes (col. 4, lines 12-15 and lines 57-63). However, rhizosecretion and guttation require specialized plant structures that are lacking in moss and liverwort plant protonema. Protonema have no roots (See, e.g., *Physcomitrella* reference of record). Instead, protonema are photoautotrophic tissue. Without roots, protonema are not capable of rhizosecretion. Protonema also do not have hydathodes, which are a particular variant of stomata (leaf opening). When exposed to copious watering and high air humidity, higher order plants that have roots will exude water through special leaf tip cells, hydathodes in the leaves, forming drops of water in the process known as guttation (See en.wikipedia.org/wiki/Guttation, last downloaded May 16, 2006, one page, attached herewith). Because root pressure provides the impetus for this fluid flow through the vessel system of the plant, in my opinion, a person of ordinary skill in the art would

have no motivation to apply the teachings of the Raskin Patent, which pertains to higher order plants having vessels and roots, to lower plants such as mosses and liverworts, which neither have vessels nor roots.

48. In sum, while the present invention involves the secretion of heterologous proteins from stably transformed protonema, which have cell walls into the culture medium, it is my opinion that a person of ordinary skill in the art would not derive sufficient guidance, or motivation, from the art of record to combine a method of the Houba-Hérin article with one or more methods of the Reutter article, the Nasu article and the Raskin Patent. In addition, it is my further opinion that even if a person of ordinary skill in the art made such a combination of the referenced teachings, the combination would not include any teaching or suggestion that there would be secretion of a heterologous protein through the cell wall of producing protonema tissue.

Summary

49. It is my opinion, based on the materials and evidence I have considered, that:

a. the state of the art regarding the cultivation and transformation of bryophytes, both mosses and liverworts, is mature and well developed, such that a person of ordinary skill in the art would have been able to make and use the method of the present invention at the time it was made to transform and culture *Physcomitrella patens*, other *Physcomitrella* subspecies, *Ceratodon* species, *Marchantia* species, and many other species of bryophytes as well because bryophytes are simple, primitive plants that are expected to behave biologically in a relatively uniform manner;

b. the experimental evidence provided by the Gorr Declaration clearly demonstrates that secretion of recombinant proteins (heterologous proteins) into cell culture medium, as described by the method of U.S. Patent Application No. 10/089,450 for *Physcomitrella patens*, can be applied to a broad spectrum of other, stably transformed bryophytes, including both mosses and liverworts, with no more than routine experimentation commonly encountered in the art;

c. with respect to the Wands factors, (i) the direction and guidance provided by the specification of the above-captioned application is sufficient so that a person of ordinary skill in the art may make and use the invention of claims 1, 17 and 19

without undue experimentation, (ii) the specification provides a detailed working example, (iii) the nature of the invention involves relatively simple, predictable plant organisms, (iv) the state of the art is mature, (v) the relative skill of those in the art is high and includes an advanced degree in cellular and molecular biology, or its equivalent, and some degree of postdoctoral training and experience, (vi) the predictability of the art is relatively high for a biological art, (vii) the quantity of experimentation necessary to practice the claimed invention is no more than is routinely encountered in the art, and is not undue, and (viii) the invention of claims 1, 17 and 19 is not overly broad;

d. each of the Wands factors weighs in favor of enablement, thereby leading to the conclusion that claims 1, 17 and 19 (copies of which are attached) are enabled;

e. the scope and content of the teachings of the Houba-Hérin article is limited to teaching a method for transiently transforming moss protoplasts, and the reference does not teach, or suggest, how to create stably transformed bryophyte strains, and it does not teach, or suggest, how to obtain secreted heterologous protein from mature protonema without disrupting producing tissue or cells;

f. the scope and content of the teachings of the Reutter article is limited to teaching a method for stably transforming moss protoplasts so as to produce intracellular heterologous protein; however, the reference does not teach, or suggest, how to obtain secreted heterologous protein from mature protonema without disrupting producing tissues or cells; and

g. a person of ordinary skill in the art would have no motivation to combine the subject matter of the Houba-Hérin article with one or more methods of the Reutter article, the Nasu article and the Raskin Patent because (i) the amount of CKO enzyme produced by the method taught by the Houba-Hérin article is so low as to have no practical value, (ii) the state of the art related to plant material comprising cell walls such as callus tissues suggested that a compartmentation could exist in the cells that keep the glycosylated form in the cell wall or plasma lemma and the unglycosylated

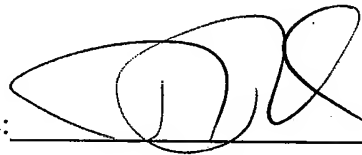
form in an internal compartment, (iii) neither the Houba-Hérin article, the Reutter article, nor the Nasu article, teach, or suggest, the application of transit peptide, and (iv) the Raskin Patent relates to higher order plants that exude water from roots and hydathodes, which are structures not present in protonema-forming plants.

50. I declare under penalty of perjury that the foregoing is true and correct, that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed by,

Date: 23.08.2006

Name: _____

A handwritten signature in black ink, appearing to be 'Ralf Reski', written over a horizontal line.

Ralf RESKI

Prof. Dr. rer. nat. Ralf Reski

Curriculum Vitae

Born in Gelsenkirchen, Germany, on 18th November 1958, married, two children.

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| today | 122 publications |
| 2004/2005 | Organiser of the International Conference on Moss Molecular Biology in Freiburg and Brno |
| 2004 | Member of the International Moss Genome Sequencing Consortium |
| from 2004 | Member of the "Ecole Supérieur de Biotechnologie Strasbourg (ESBS) |
| from 03.2004 | Member of the scientific advisory board of the BMBF (German federal ministry of science) Project "Safety assessment of transgenic plants" |
| 2003-2006 | Member of the Habilitation-committee of the Institute Biology, Freiburg University |
| from 17.01.2003 | Advisory Board of BioPro GmbH, an enterprise of the federal state of Baden-Württemberg. |
| from 14.10.2002 | Managing Director of the Institute Biology II, Freiburg University. |
| 2002-2005 | Chairman of the advisory board at greenovation Biotech GmbH. |
| 2002-2005 | Member of the FESPB Grants and Awards Committee |
| since 14.07.2001 | Life Science co-ordinator of Freiburg University. |
| since 01.03.2001 | Director Plant Biotechnology of the Center for Applied Biosciences at Freiburg University. |
| since 04.11.1999 | Scientific co-ordinator of the BioRegio Freiburg. |
| since 21.12.1999 | Full Professor (C4), Chair Plant Biotechnology, Freiburg University. |
| 09.09.1999 | Co-founder of greenovation Plant Biotechnology GmbH. |
| since 13.04.1999 | Co-ordinator of the German National Science Foundation (DFG) Program "Molecular Analysis of Phytohormone Action". |
| 19.03.1999 | Rejection of an offer for a chair "Cell and Molecular Biology of Plants" at the Technical University Dresden. |
| 01.03.1999 | Professor for Plant Biotechnology at Freiburg University. |
| 1999-2005 | Co-operation with BASF AG on plant functional genomics (more than 10 Mio. Euro) |
| 14.05.1997 | Rejection of an offer for an Associate Professorship at Oslo University (Norway). |
| 1996-1999 | Heisenberg-Fellow of the German National Science Foundation |

(DFG) at Freiburg University.

04.09.1996 Rejection of an offer to substitute as a Professor of Botany at Kiel University.

06.12.1994 Habilitation awarded in "General Botany".

01.04.1990 Assistant Professor (C1) at Hamburg University, Department of Cell Biology.

30.03.1990 Ph.D. Degree (Dr. rer. nat.) at Hamburg University, Department of Genetics.

Studies in Biology, Chemistry, Theory and Practice of Education at Gießen University and Hamburg University.